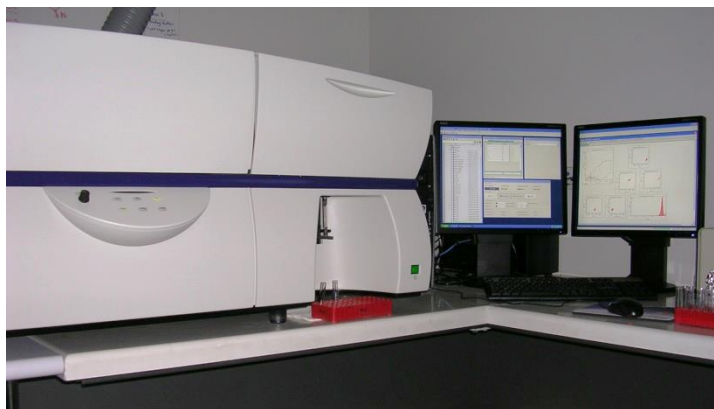


Facility Contact Details

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Important Points:

- If a problem arises which you are unsure how to resolve, please do not try and fix it yourself – Please contact a member of the AMREPFLOW Staff (Leave a note on the instrument and email facility staff if things go wrong outside of business hours)
- All users **MUST** be licensed to use the instrument and be familiar with the policies of AMREPFLOW. Disregarding SOPs or abuse of systems may result in a suspension of instrument use. To become licensed please contact AMREPFLOW staff.
- Correct shutdown procedure must be adhered to. Failure to do so can result in instrument blockages and malfunction, impacting the work of the next user.
- FACS DiVa Software guide is available on the computer desktop. Please refer to this document for comprehensive guidelines on how to use the software. If further assistance is required please contact a member of the AMREPFLOW staff.

Sample Preparation

1. The LSRII is equipped with 405nm, 488nm, 561nm and 633nm lasers, permitting detection of signals in 16 fluorescent channels. Before designing an experiment or purchasing an antibody, please ensure it is compatible with this instrument.
2. Instrument specifications are available at www.amrepflow.org.au.
3. We have some additional optical filters that may be better suited to your work, furthermore please ask for assistance if your panel could be better optimized.
4. The core facility recommends using FluoroFinder (<https://app.fluorofinder.com/amrep/panels/new>) for custom panel design specific to AMREPFLOW instruments.
5. **ALL** samples **MUST** be filtered through a (minimum) 70µm mesh filter before acquisition – **NO EXCEPTIONS**
6. All samples must be resuspended at an appropriate cell concentration to not block the Sample Injection Probe (SIP).
 - Recommended cell concentration is 1×10^6 cells/mL (equivalent to 2×10^5 cells/200µL)
7. Users who continue to block the SIP through neglectful sample preparation may have their usage privileges revoked.

Instrument Start-up

1. Locate the green power button on the right hand side of the instrument; press to switch on the LSRII. This will warm up the lasers and begin to pressurise the fluidic system. Some buttons on the fluidics panel, on the front of the instrument, should be illuminated indicating it is ON.
2. Switch on the desktop PC and login to Windows. Login information is supplied underneath the monitors.
3. Once logged into Windows, proceed to open and login to BD FACSDiVa software.
 - a. **UNDER NO CIRCUMSTANCES ARE LOGIN DETAILS TO BE SHARED. SHARING OF THESE DETAILS OR LOGGING IN FOR ANOTHER USER WILL NOT BE TOLERATED AND WILL RESULT IN RESTRICTED INSTRUMENT ACCESS; STRICTLY LICENSED USERS ONLY.**
 - b. Use the DiVa login that was individually created for you, if you do not have a profile on the instrument, please contact a staff member to rectify this.
 - c. Be prepared to wait for the instrument to connect and communicate with the PC, this may take approximately five minutes. Even if you are planning to use tubes instead of the HTS, you will have to wait for the HTS to initialise before DiVa is available.
4. After logging into DiVa and waiting for the cytometer to connect, you may be prompted with a warning about "CST Mismatch".
 - a. Select "Use CST Settings", as these settings will use the most up to date time delay calculations for the instrument. Should you not be asked to use CST settings, the instrument will already use these settings.
5. Proceed to check the fluidics.

Fluidics

Sheath

- The LSR II uses 18.2MΩ MilliQ water as sheath fluid. This is provided in 20L containers in the lab; generally located near the sink.
- The Sheath tank is the stainless steel vessel. It must only be filled with MilliQ water to $\frac{3}{4}$ of the tank's total volume (to the upper seam line), this is enough sheath to run the instrument for approximately 4 hours.
- There is no sensor to indicate the remaining volume; users must be vigilant to check the level of sheath fluid before they begin acquisition by opening the tank and checking.
- **Users are expected to fill up the Sheath tank for the subsequent user at the end of their session.**

Filling the Sheath tank

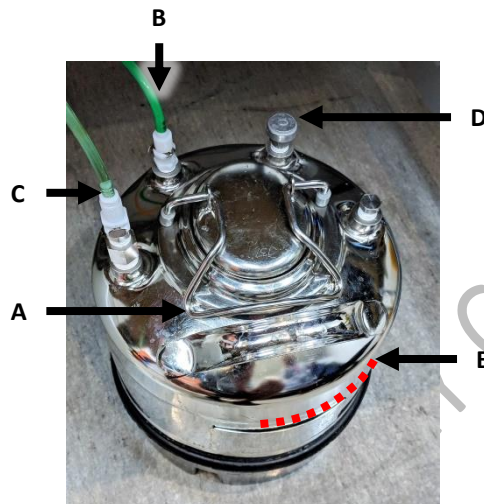


Figure 1: Stainless steel sheath tank

1. Raise the thin handle (1A) to loosen lid.
2. **DO NOT** remove the air intake (1B) or fluid (1C) lines from the sheath tank, but check to see if they are securely connected.
3. Depressurise the vessel by pulling up on the pressure valve (1D) for a few seconds until the pressure has been relieved and you no longer hear any hissing.
4. The lid should drop on its own accord once the tank is depressurised, if not you may need to push down on the lid to be able to overcome the seal.
 - a. The instrument will constantly pressurise the system if it is ON.
5. Remove the lid and fill the sheath tank with MilliQ water to the upper metal seam line in the tank (1E). This will ensure the tank is roughly $\frac{3}{4}$ full.
6. Reinsert the lid and rotate so it fits snugly in its place (it will only fit in one way). Lower the handle of the lid, so it comes into contact with the tank to seal the sheath tank.
7. Wait approximately 30 seconds until the sheath tank is pressurised, then remove any air from the sheath lines.

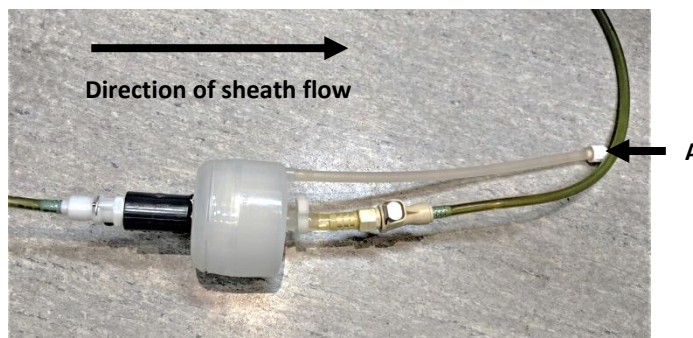


Figure 2: Inline sheath filter

- a. Bleed any air bubbles by removing the bung (2A) on the inline sheath filter located under the bench and drain the water and any air bubbles into a supplied container. This will expel sheath fluid at moderate pressure, so ensure that the line is directed into the container and any spills are mopped up.
 - b. Another valve to be bled is located on the side of the instrument. Use the rolling-valve to bleed any air from the sheath line before it enters the instrument.
 - c. FAILURE TO COMPLETE THESE STEPS CAN RESULT IN POOR OR NON-EXISTENT SIGNALS FROM THE 405, 561 AND 640nm LINES.
8. Ensure the instrument is in Tube-mode operation.
 - a. On the right hand side of the instrument is a small switch near the Power button.
 - b. Flick the switch to an “Up” position to enable Tube-mode.
9. Prime the instrument twice at the beginning of your session.
 - a. Remove any tube that is currently on the SIP by pushing the arm to one side and gently pulling on the tube to remove it.
 - b. Direct yourself to the Fluidics panel to the left of the SIP and press the PRIME button.
 - c. The system will initiate a ~30 second “flush-and-fill” of the flow cell, removing any residual air and debris from the sample intake line and flow cell, before returning to STANDBY mode.
 - d. Repeat the PRIME procedure again.
 - e. Note: Priming with a tube installed on the SIP is acceptable, but please be aware any particulate matter will be ejected into the tube, subsequent acquisition of the same tube will involve acquisition of this debris. Please do not reuse these same tubes for cleaning, dispose and replace with new tubes.
10. Return a clean water tube on to the SIP and place the instrument either in STANDBY or RUN on HIGH until you’re ready to begin cleaning or sample acquisition.

Waste

- The waste tank holds a higher volume of liquid than the sheath tank. Do not use the waste tank volume as an indicator of remaining sheath. Periodically check the volume of sheath in the sheath tank to ensure the sheath does not run empty.
- At the end of your session, users are expected to empty the waste down the sink, and add (minimum 200mL) 5% bleach to cover the bottom of the waste tank.

Emptying the Waste tank




Figure 3: LSRII waste container

1. Waste tank should be emptied when volume of liquid extends more than 3cm from base of tank (3A).
2. **DO NOT** use the silver quick-connect tab to remove the orange waste line (3B).
3. Rotate the drum and not the lid to disconnect the waste container.

4. Take the waste container to the sink and pour the contents of the waste tank down the sink whilst simultaneously running tap water to flush and dilute any excess hypochlorite down the sink.
5. Once the tank is emptied, add (minimum 200mL) 5% Sodium Hypochlorite to the tank.
 - a. Please ensure you are wearing gloves, safety glasses and a lab coat for this step (basic PC2 biosafety).
 - b. Sodium Hypochlorite is caustic and could damage your eyes, irritate skin and will damage your clothing should you splash or spill it on yourself (be familiar with the MSDS in case of spillages).
6. Return waste tank to cytometer and replace lid, again by rotating the drum and not the lid.
 - a. Do not overtighten the lid, this should remain loose to avoid backpressure issues.
7. Ensure waste line connections are tightly in place with no loose junctions, and avoid pulling the waste line so that it is taut.

DiVa Software Navigation and Sample Setup for a new experiment

- DiVa operates with multiple windows in a larger 'shell'.
 - a. Browser: This window is where experiments are organised.
 - i. Creation of new experiments is achieved through the icons at the top of the Browser window, or within the menu from the toolbar.
 - ii. Right-click on an open experiment (the book icon appears open) to Rename the experiment. Please rename the experiment with a suitable identifier (i.e. Immunophenotyping, GFP expression, NK cell Panel, Cytometric Bead array, Cell Cycle Assay etcetera).
 - iii. Beneath the Experiment level, a Specimen needs to be created if not automatically done.
 - iv. Right-click and rename the Specimen with the date in the Year-Month-Day (YYYYMMDD) format; this will make it easier to locate files when they are exported.
 - v. Samples to be acquired during this particular date/session should be located beneath/within the Specimen by clicking on the "+" icon next to the Specimen name to open a subsection with a prepopulated tube (i.e. Tube_001).
 - vi. Tubes should be renamed to identify the contents of the sample to be acquired (Right-click > Rename).
 - vii. A tube needs to be 'active' by clicking on the "  " icon to the left of the tube name. When green, this will activate the remaining windows (a yellow-coloured icon, indicates active, electronic event acquisition).
 - viii. New tubes can be created from the toolbar at the top of the Browser window or within the Acquisition Dashboard by clicking "Next Tube" (recommended).
 - b. Cytometer: Contains multiple tabs for instrument settings.
 - i. The "Status" tab is coloured red in the event of an instrument error; please notify staff if this occurs.
 - ii. The "Parameters" tab is where instrument parameters/channels can be added/removed and where PMT voltages can be adjusted. Ensure the Height (H) and Width (W) parameters for FSC and SSC are ticked.
 - iii. The "Threshold" tab provides options for adjusting the electronic threshold of the instrument. Please ask staff if you would like help with adjusting the threshold.
 - iv. The "Laser" tab provides the user access to laser delay and scaling settings. Users are advised not to adjust these values.
 - v. Removal of spectral spill-over can be done in the "Compensation" tab.
 - vi. The "Ratio" tab is generally used for certain kinetic assays.
 - c. Inspector: Provides the user with more options depending on the selected object.
 - i. Users can customise certain aspects of the selected worksheet, plots, experiments, specimens, tubes etcetera by accessing the settings in this window.
 - d. Acquisition Dashboard: Controls options for event collection and recording of data.
 - i. If plate controls are required, right-click on the dashboard to display a plate-specific menu and options.
 - e. Worksheet: Workspace for the creation and viewing of cytometry plots and statistics.
 - i. Ensure the "Global Worksheet" is being used and not the "Normal Worksheet". To change this, click the page icon in the toolbar to switch between modes.

- ii. PDFs of the worksheet can be saved by clicking on the PDF icon. Ensure worksheet elements are on the white space to ensure they aren't clipped when creating PDFs. (To see page breaks on the worksheet; click the worksheet and navigate to the Inspector window, check the box to Show Page Breaks.)
 - iii. Plot and Gating options are available along the top window toolbar.
1. Create a new Experiment, book icon, in the Browser window.
2. Rename the experiment with the type of work or the panel you're using.
3. Create a new Specimen (if it is not automatically generated), syringe icon, and label with the date using YYYYMMDD format.
4. Click on the "+" icon next to the Specimen name/Date, to expand the item, presenting you with a blank tube. Each sample will be acquired as its own individual tube.
5. Click on the "D" icon to the left of the Tube to activate the tube and remaining windows.
6. Rename the Tube to reflect the contents of the sample you'll be acquiring.
7. Go to the Cytometer window, Parameters tab, to enable recording of the Height and Width (H and W) elements of FSC and SSC.
8. Remove any unnecessary or unused fluorescent parameters by selecting the parameter and clicking the Delete button within the Cytometer window. Use the Add button or parameter drop-down list to add any parameters that have been erroneously deleted.
9. Go to the Compensation tab in the Cytometer window and check the Enable Compensation checkbox.
 - a. With the Tube selected in the Browser, move to the Inspector window and select the Labels tab to input marker and fluorochrome information if required.
10. Right-click on Cytometer Setting in the Browser window, in the subsequent menu select Application Settings > Create Worksheet. A second Global Worksheet with a number of prepopulated plots will be prepared in the Worksheet pane.
 - a. Create any histograms or bivariate plots required for setting manual compensation.
11. Load your unstained control and ensure cells are visible within the FSC vs SSC plot.
 - a. Ensure you are looking at the correct events and not debris/noise.
 - b. Adjust the FSC and SSC PMT voltage to fit negative population into "grey areas" indicated on the plots, as suggested by Application Template.
 - c. Ideally use cells or particles for setup; unstimulated T, B, NK, RBCs or beads with nominal autofluorescence. (N.B. Beads will manifest 0.5-1 log higher off the violet line in emissions between 450-600nm.)
 - d. Always save your controls.
12. Remove the unstained control and click "Next Tube" in the Acquisition dashboard.
13. Load a fully-stained sample.
 - a. Acquisition of the fully stained sample is to ensure voltages are not too high and won't cause any signal to be off-scale for the duration of your sample acquisition.
 - b. Acquire this sample at a low flow rate to avoid running too much of your experimental sample.
 - c. Adjust any voltages for the fluorescent parameters if they appear off-scale.
 - d. Do not touch the fluorescent parameter voltages after this has been completed. Doing so will mean Compensation controls will have to be re-acquired. Voltages need to be the same for each sample for compensation to be relevant.
14. Remove the fully-stained sample, allow the instrument to backflush momentarily.
15. Load your first single colour control and begin sample acquisition.
 - a. Ensure sample is showing signal in the correct channel.
 - b. Record a suitable number of events to properly compensate.
16. Proceed with compensation.
 - a. On a bivariate plot, place the source (Fluorochrome in the single colour control) on the X-axis, and the detector (used to measure spillover) on the y-axis.
 - b. Compensate by removing the spillover belonging to the positive population out of the detector.
 - c. Aim to adjust the spillover such that the positive population has a Median Fluorescence Intensity equal to that of the negative population in the detector (y-axis) you're looking at.
17. Click "Next Tube" in the Acquisition Dashboard to carry-over the compensation and voltage settings from the current tube, into a new tube.
18. Continue with steps 15-17 for the remaining single colour controls for compensation.
 - a. Ensure all Compensation Controls have been saved.

19. Proceed with acquiring and saving/recording data for your experimental/fully-stained samples.
 - a. Ensure the Stopping Gate and Events to Record (in Acquisition Dashboard) are set appropriately, so that you are collecting enough relevant events for your work.
 - b. Do not adjust the Storage Gate; always leave this on All Events.
20. After acquisition of final sample, proceed with cleaning the instrument for the next user.

Sample Acquisition using Tubes

- Refer to the right-hand side of the instrument to ensure the switch is engaged correctly for tube acquisition (i.e. Up!).
 - Ensure that tubes can be attached to the SIP without assistance of the tube support arm underneath. This may require tightening the nut at the top of the SIP.
 - Tubes should be of the polystyrene variety and should fit snugly around the nut of the SIP. Forcing a tube on to the SIP can risk moving the flow-cell.
 - Cracked tubes will not pressurise; contents will need to be transferred to a new tube before sample acquisition can take place.
 - The SIP has been modified so that it constantly backflushes with sheath when the arm is aside; irrespective of whether or not a tube is on the SIP.
 - Thoroughly resuspend or vortex cells before sample acquisition.
 - If there are any visible clumps (when held up to a light), cells need to be passed through an appropriate cell strainer or similar to prevent blockages and sample acquisition can proceed.
1. Load sample on to SIP and realign arm underneath the tube.
 2. At the fluidics panel, start acquisition by setting the flowrate to “Low” and pressing “Run”.
 - a. Run button should appear a solid green, if not, the instrument isn’t pressurised correctly for delivery of sample into the cytometer.
 - b. When Run is selected, the cytometer is actively taking up sample into the instrument.
 3. Return to the computer and select “Acquire” in the Acquisition Dashboard to begin seeing events in plots on the screen/s.
 4. Ensure cells are appearing and are in an appropriate position in FSC and SSC plots.
 - a. Adjust voltages if necessary to ensure cells are on-scale (only for FSC and SSC).
 5. Confirm or adjust voltages for fluorescent parameters using any appropriate controls (unstained or fully stained sample).
 6. Acquire your samples ideally using the same flow rate, avoid exceeding >6000 events/second.

Instrument Cleaning and Shutdown

Shut-down procedure after Tube acquisition

1. After running of your final sample, move the support arm to the side, remove the tube and allow between 5-10 drops of sheath to backflush from the SIP.
2. Place a tube of MilliQ water on the SIP and allow it to RUN on HI flowrate for 5 minutes.
3. Repeat step 2 using 5% hypochlorite (Bleach) solution for 5 mins.
4. Repeat step 2 using MilliQ water to rinse any residual hypochlorite from the instrument.
5. Log out of FACSDiva after exporting your data and checking to see if you’re the final user of the day.
6. Leave a tube containing ≥ 1 mL of MilliQ water on the SIP and set the instrument to STANDBY.
7. Turn off cytometer using the big green button and shut down the computer.
 - a. The cytometer should be left ON only if there is a user scheduled to use the instrument within the next hour.

Exporting Data

1. Users should get in the habit of transferring their data to a suitable storage medium at the conclusion of their experiment.
2. Navigate to the Browser window and select the Experiment or the Date (Specimen level).
3. Right-click to open a menu and scroll down to Export.
4. Select FCS files to export your raw data from the DiVa software to a location on the computer, preferably Desktop.
5. Specify the save location in the subsequent windows before clicking Save to initiate the export process.
 - a. Be patient. Interrupting this step, especially with large files can result in corrupted data.
6. Connect your suitable external storage medium (USB, external hard drive etc.).
7. Cut and Paste your files to your storage medium.
8. Safely disconnect your storage device from the computer.
9. Ensure you store your data securely and that appropriate backups are in place.

Document Revision History

Date	Version	Edits/Comments	Author (Initials)	Approved (Date and Initials)
2013	1.2			
20190417	1.3	Updated for LSR II	SL	GP 20190417